H. A. L. Wahdan

Causes of the Antimicrobial Activity of Honey

Summary: The present study was performed to clarify the possible causes of the antimicrobial activity of honey. A sugar solution resembling honey in its high sugar content was made. The antimicrobial activities of both honey and this solution towards 21 types of bacteria and two types of fungi were examined. The results achieved by both were compared. The difference between them indicated the presence of antimicrobial substance(s) in honey. The kinds of antimicrobial substances (inhibines) in honey are discussed. Hydrogen peroxide is not the only inhibine in honey. In fact, inhibines in honey include many other substances. Two important classes of these inhibines are the flavonoids and the phenolic acids. Flavonoids have often been extracted from honey previously. In this study two phenolic acids (caffeic acid and ferulic acid) were extracted from honey for the first time.

Introduction

Since the antimicrobial activity of honey was first described by *Dold* et al. [1], it has been discussed by many authors [2–5]. The known causes of the antimicrobial activity of honey are the high osmolarity [4, 6], acidity [4], hydrogen peroxide [7] and unidentified substances from certain floral sources [6]. Besides demonstrating antimicrobial activity of honey, this study attempts to evaluate the effects of the high osmolarity, the consequence of the acidity and the type of antimicrobial substances in honey.

Materials and Methods

The honey used was trefoil honey from the Faculty of Agriculture, Cairo University. Fructose and glucose were bought from El-Gomhoria Company for Drugs and Chemicals. Standards, caffeic acid and ferulic acid, were purchased from SIGMA. A sugar solution was prepared containing the following: 46.5% fructose, 34% glucose, 1.5% sucrose and 18% water.

Bacterial broth dilution method: Decreasing concentrations of honey and sugar solution were each prepared in serial twofold dilutions using nutrient broth. A standard inoculum of the microorganism (e.g., organisms 1×10^6 ml, a 1:500 dilution of a suspension of turbidity equal to a McFarland standard 1.0) was added to an equal volume (1 ml) of each concentration and to a tube of the growth medium without honey or the sugar solution that served as a growth control. An uninoculated tube of nutrient broth was incubated to serve as a negative growth control. After overnight incubation, the tubes were examined for turbidity indicating growth of the microorganism. The lowest concentration each of honey and sugar solution that inhibited growth of the microorganism as detected by the lack of visual turbidity (matching the negative growth control) was designated the minimum inhibitory concentration (MIC).

The bactericidal activity of honey and sugar solution was tested as follows: The number of the bacteria in the initial microorganism suspension was counted by the surface plate method [8]. After ascertaining the MIC, the number of bacteria was counted in each of the tubes of broth that showed no visible turbidity after overnight incubation, and was compared with the number of bacteria in the initial microorganism suspension. According to [9], the lowest concentration each of honey and sugar solution that allowed less than 0.1% of the original inoculum to survive was taken to be the minimum bactericidal concentration (MBC).

For sensitivity testing of fungi, the solid media used were Sabouraud dextrose agar for *Candida albicans* and Malt extract agar plus 1% actidione and 1% chloramphenicol solutions for *Trichophyton mentagrophytes* and the incubation periods were 2 days at 37°C for *C. albicans* and 10 days at 30°C for *T. mentagrophytes*.

Sample preparation for chromatography: The method used was a modification of the methods described as suitable for the separation of phenolic acid from honey [10, 11]. Honey was diluted with distilled water 1:5 to give a 20% solution.

Preparation of tube 1: In 50 ml of this honey solution (= 10 ml honey), pH was adjusted to 3.5 by 4N HCl. Fifty ml ethyl acetate and approximately 1 gram of dry sodium bisulfite were then added in a separating funnel. The mixture was shaken for 5 min to extract the phenolic compounds. The ethyl acetate layer was poured into a beaker and another 50 ml ethyl acetate was added to the honey solution layer. The above steps were repeated so that the honey solution was extracted six times. The 300 ml ethyl acetate extract was concentrated in a rotary evaporator under vacuum at 30°C to about 10 ml. This concentrate was taken up in methanol in a sterile tube, dried under nitrogen and stored at 0°C.

Preparation of tube 2: As it was expected that some phenolic acids were bound to glucose as esters, another sample preparation was made to liberate these bound phenolic acids. Twenty-five ml of the honey solution (20%) were added to 25 ml 3N NaOH and stored under nitrogen at room temperature for 4 h to hydrolyze the esters. pH was adjusted to 3.5 by 4N HCl and extraction was performed as in tube 1.

Tube 1 thus contained the free phenolic acids in 10 ml honey, whereas tube 2 contained the total phenolic acids (free and bound) in 5 ml honey.

High performance liquid chromatography (HPLC): A HPLC Perkin-Elmer system (USA) equipped with a binary LC-250 pump with constant flow rate of 0.8 ml/min and LC-290 UV/vis

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Dr. *H. A. L. Wahdan*, Food Microbiology Laboratory, Central Health Laboratories, Ministry of Health, 19 El Sheik Rihan St., El Falaky, Cairo, Egypt.

Table 1: Antibacterial activity of honey and of the sugar solution.

Bacterial species	Original bacterial counts CFU/ml	Counts CFU/ml after incubation with concentrations of Honey							Sugar Solution		
		Undiluted	1/2	1/4	1/8	1/16	1/32	1/64	Undiluted	1/2	1/4
1. *Salmonella paratypi B*	3,335,000	0	0 (MBC)	T	T	T	T	T	0 (MBC)	1,500.000	T
2. *Salmonella typhimurium*	5,275.000	0 (MBC)	15,500	T	T	T	T	T	0 (MBC)	T	T
3. *Salmonella enteritidis*	7,067.500	0	0	0 (MBC)	T	T	T	T	0 (MBC)	T	T
4. *Salmonella newport*	2.090,000	0	0 (MBC)	T	T	T	T	T	0 (MBC)	T	T
5. *Shigella flexneri*	2,700.000	0	0	0	0 (MBC)	T	T	T	0 (MBC)	T	T
6. *Shigella boydii*	900.000	0	0	0	0 (MBC)	0	T	T	0	0 (MBC)	T
7. *Proteus mirabilis*	28,410,000	0 (MBC)	39,500	T	T	T	T	T	500,000	T	T
8. *Pseudomonas aeruginosa*	100,000.000	1,000	1,000	5,000 (MBC)	T	T	T	T	0 (MBC)	T	T
9. *Yersinia ruckeri*	52.000,000	0	0 (MBC)	T	T	T	T	T	10.500	T	T
10. *Aeromonas hydrophilia*	233.000	0	0	0 (MBC)	T	T	T	T	0	0 (MBC)	T
11. *Klebsiella pneumoniae*	2,317.000	0	0 (MBC)	T	T	T	T	T	5,500	T	T
12. *Klebsiella aerogenes*	750,000	0 (MBC)	200,000	T	T	T	T	T	163,500	T	T
13. *Enterobacter sakazakii*	2,742.000	0 (MBC)	6,000	T	T	T	T	T	20,000	T	T
14. *Escherichia coli*	17,000,000	0 (MBC)	500,000	T	T	T	T	T	17.500	T	T
15. *Haemophilus influenzae*	79,700,000	0	0 (MBC)	T	T	T	T	T	155,000	T	T
16. *Vibrio cholerae O 1 (V. ogawa)*	4.000.000	0	0	0	0	0	0 (MBC)	T	0 (MBC)	T	T
17. *Corynebacterium diphtheriae*	3.620.000	65,500	T	T	T	T	T	T	430,000	T	T
18. *Staphylococcus aureus*	24,500,000	1,125,000	1,150,000	T	T	T	T	T	500 (MBC)	T	T
19. *Staphylococcus epidermidis*	52,500,000	0 (MBC)	T	T	T	T	T	T	787,500	T	T
20. Viridans *Streptococcus*	355,000	3,000	T	T	T	T	T	T	0 (MBC)	T	T
21. *Streptococcus pneumoniae*	32,400,000	0	0 (MBC)	25,500	T	T	T	T	0 (MBC)	T	T

T = Turbid tubes (not counted); (MBC) = minimum bactericidal concentration; The minimum inhibitory concentration (MIC) is the concentration before the turbid tubes (T). All concentrations greater than MIC are inhibitory concentrations and all concentrations greater than MBC are bactericidal concentrations.

detector with absorbency at 280 nm was used. The analytical column was a reversed phase (C_{18}) column of 3 μm particle size, 3.3 cm length and 0.46 cm internal diameter. The guard column was C_{18} of 10 μm particle size, 3.3 cm length and 0.46 cm internal diameter. The integrator was a LC1-100 lab-computing integrator with chart speed of 10 mm/min. HPLC was performed using mobile phases of A = water-acetic acid (99:1, v/v) and B = methanol-acetic acid (99:1, v/v) in a gradient process beginning with A = 60% and B = 40% within 2 min, then increasing B to 100% through 6 min and maintaining it for about 6 min. Standards and samples were dissolved in acetonitrile:water (4:1, v/v) before injection. Quantitation was carried out by incorporation of external standards, caffeic acid and ferulic acid.

The calibration report obtained was as follows:

Retention time	Response factor	Amount	Name
0.490	6.236088E-08	0.9390	caffeic acid
1.260	2.234568E-08	0.1179	ferulic acid

Gas liquid chromatography (GLC): The methyl esters of the samples and standard compounds were analyzed with a GCV Pye Unicam gas chromatograph equipped with dual flame ion-

Table 2: Comparisons between bacteriostatic and bactericidal activities of concentrations of honey and the sugar solution.

Concentrations	Bacteriostatic activity						Bactericidal activity					
	Honey		Sugar solution		P	S'	Honey		Sugar solution		P	S'
	No. (T = 21)	%	No. (T = 21)	%			No. (T = 21)	%	No. (T = 21)	%		
Undiluted	21	100%	21	100%	-	-	18	85.7%	12	57.1%	< 0.01	S
1/2	18	85.7%	3	14.3%	< 0.001	S	12	57.1%	2	9.5%	< 0.001	S
1/4	7	33.3%	0	0%	< 0.01	S	6	28.6%	0	0	< 0.01	S
1/8	3	14.3%	0	0%	< 0.05	S	3	14.3%	0	0	< 0.05	S
1/16	1	4.8%	0	0%	> 0.05	N	1	4.8%	0	0	> 0.05	N
1/32	1	4.8%	0	0%	> 0.05	N	1	4.8%	0	0	> 0.05	N

No. = number of bacterial species ; T = total number of bacterial species; S' = significance; S = significant; N = not significant; P = probability. Honey had no bactericidal concentration with three species of bacteria (14.3%), whereas the sugar solution had no bactericidal concentration with nine species (42.9%).

Table 3: Activity of honey and the sugar solution against two species of fungi.

Type of fungus	Original fungal counts CFU/ml	Counts CFU/ml after incubation with concentrations of			
		Honey		Sugar solution	
		Undiluted	1/2	Undiluted	1/2
1. *Candida albicans*	8,350,000	1,045,000	T	T	T
2. *Trichophyton mentagrophytes*	2,000	40	T	T	T

A colony-forming unit of *Trichophyton mentagrophytes* was added directly to each of 1 ml of honey and the sugar solution and the test was repeated. After incubation honey was clear, whereas the sugar solution was turbid. The fungal count of honey was 0/ml.

ization detector. The fractionation of the methyl esters was conducted using a coiled glass column (2.8 m X 4 mm) packed with Diatomite C (100–120 mesh) and coated with 1% OV-17. The oven temperature was programmed at 10°C/min from 70°C to 270°C, then isothermally at 270°C for 25 min. The nitrogen flow rate was 30 ml/min. Detector and injector temperatures were 300°C and 280°C. Hydrogen and air flow rates were 33 ml and 330 ml/min, respectively. Peak identification was performed by comparing relative retention time of each compound with those of the standard materials using the PU 4810 computing integrator (Philips). The standard calibration report was as follows:

Retention time	Name
14.88	caffeic acid
16.67	ferulic acid

Statistical analysis: The statistical analysis was performed by using the Z-test for the difference between two proportions according to SYSTAT [12].

Results

The Antimicrobial Activities of Honey and the Sugar Solution

Table 1 lists the antibacterial activities achieved by honey and the sugar solution against 21 species of bacteria. Table 2 collects the data of Table 1 comparing the bacteriostatic and bactericidal activities of each concentration of honey and the similar concentration of the sugar solution. There were significant differences between the bacteriostatic activities of the diluted forms (1/2, 1/4, 1/8) of both and between the bactericidal activities of the undiluted as well as the diluted forms (1/2, 1/4, 1/8). Table 3 shows the antifungal activities of honey and the sugar solution towards *T. mentagrophytes* and *C. albicans*.

HPLC

Caffeic acid was separated from tube 1 (Figure 1), whereas caffeic acid and ferulic acid were separated from tube 2 (Figure 2).

GLC

Caffeic acid and ferulic acid were separated from tube 1 (Figure 3) and tube 2 (Figure 4).

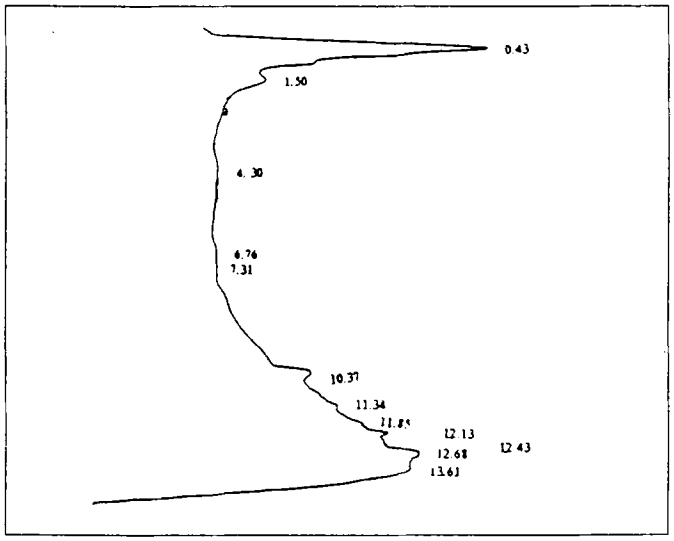

Figure 1: Sample 1, tube 1, HPLC.

Retention time	Area	Amount (mg)	Name
0.43	8,834,752	0.08834752	Caffeic acid

One matched component and 11 unknown peaks.

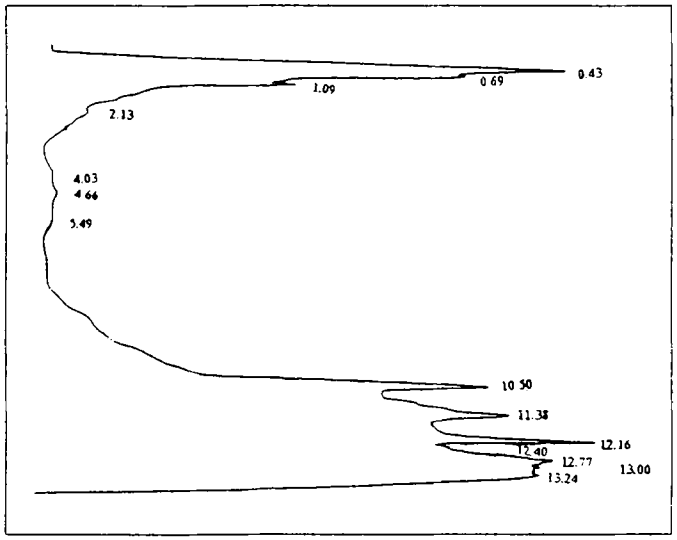

Figure 2: Sample 1, tube 2, HPLC.

Retention time	Area	Amount (mg)	Name
0.43	3,974,001	0.03974001	Caffeic acid
1.09	2,771,964	0.02771964	Ferulic acid

One matched component and 13 unknown peaks.

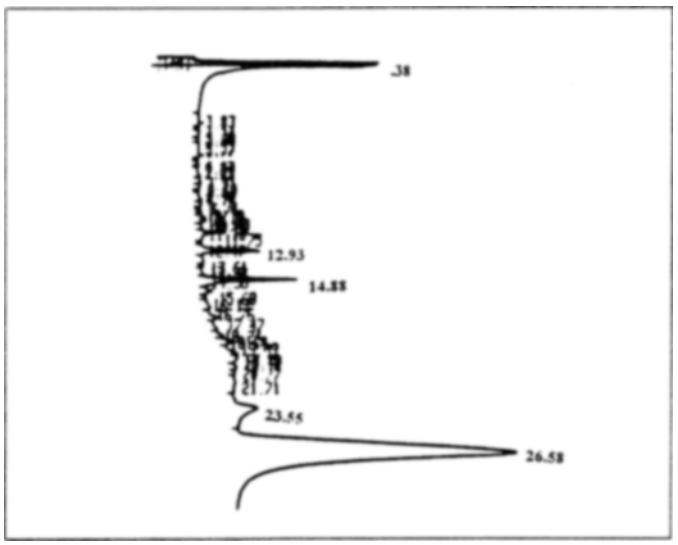

Figure 3: Sample 2, tube 1, GLC.

Retention time	Area	Name
14.88	3,751,000	Caffeic acid
16.76	228,870	Ferulic acid

Two matched components and 17 unknown peaks.

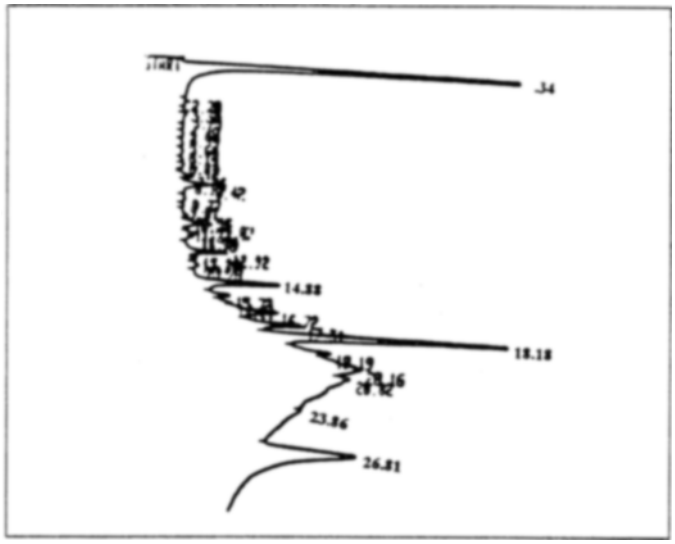

Figure 4: Sample 2, tube 2, GLC.

Retention time	Area	Name
14.88	5,626,300	Caffeic acid
16.72	7,900,900	Ferulic acid

Two matched components and 26 unknown peaks.

Discussion

The Antimicrobial Activity of Honey

The antibacterial activity of honey was achieved mainly by the high concentrations (Tables 1 and 2). This suggests that the most beneficial use of honey in treatment of bacterial infections is when it can be applied directly to the bacteria without much dilution by body fluids or other factors as in bacterial skin diseases, septic wounds and eye infections. This agrees with clinical research on the use of honey in the treatment of wound sepsis and burns.

Hamdy et al. [13] showed that honey cleared up superficial septic wounds better than Savlon antiseptic. *Gupta* et al. [14] demonstrated that experimentally induced cutaneous infected wounds in buffalo calves healed significantly faster with natural honey than ampicillin ointment and honey-ampicillin mixture. Honey was more efficient than silver sulfadiazone gauze dressing in treatment of superficial burns [15], and burns treated with honey-impregnated gauze healed faster than those treated with amniotic membrane dressing [16].

The antifungal activity of honey towards *T. mentagrophytes* and *C. albicans* in the present study conforms to the well-documented antifungal properties of honey [17]. This suggests that undiluted honey can be used in the treatment of superficial fungal infections such as ringworm disease and superficial candidiases.

Honey, however, may contain spores of *Clostridium* and *Bacillus* spp. which may lead to exposure of the patient to additional risk of infection such as wound botulism or gangrene [7]. Honey must be sterilized before using it as a therapeutic. Sterilization of honey can be accomplished by cobalt 60 gamma radiation [18].

Causes of the Antimicrobial Activity of Honey

The high sugar concentration: Honey is characterized by high sugar and low water concentrations. The total content of reducing sugars in honey comprise 69.6% [19], 69.5% [20]; or 67% in winter honey, 75% in honey produced in other seasons and up to 80% in wild honey [21].

Fructose concentration in honey may be between 36.9 and 47.3% [19, 21–23]. Glucose concentration may be between 27 and 34.9% [21–23]. Sucrose concentration may be between 0.2 and 2.7% [20, 23]. Maltose concentration may be between 0.7 and 11% [19, 20]. Melezitose concentration may be 0.6% [19].

The water content of honey may comprise 12.4–20.3% [20]; 19.9–20.3% [23]; or 16.3–18.5% [26].

In this study, I made the sugar solution mimic this property of high sugar concentration in honey which leads to the high osmolarity that produces antimicrobial action. The antimicrobial activity of this solution was compared with that of honey using the same microorganisms.

Since the sugar solution resembled honey in its high sugar content, the bacteriostatic and bactericidal activity accomplished by its undiluted form (Tables 1 and 2) indicate the importance of the high sugar concentration in the antibacterial activity of honey.

As recorded in Table 2 the significant differences between the bacteriostatic activities of the diluted forms (1/2, 1/4, 1/8) of honey and the sugar solution and between the bactericidal activities of the undiluted as well as the diluted forms (1/2, 1/4, 1/8) indicate the presence of other contributory cause(s) of the antibacterial activity of honey.

The absence of inhibitory activity of the sugar solution towards *T. mentagrophytes* and *C. albicans* speaks for other factor(s) causing the antifungal activity of honey.

pH of honey: This may be a second possible reason for its antimicrobial activity. pH of honey may range from 3.77 to 4.01 [24]. In general, molds can grow in lower pH than yeasts. For example, the minimum pH for growth of *Aspergillus niger* is 1.2 and for growth of *Aspergillus oryzae* is 1.6–1.8 [25]. Yeasts can grow in lower pH than bacteria. For instance, the minimum pH for growth of *C. albicans* is 2.2. There is a wide range of the minimum pH for the growth of the bacteria. For illustration, the minimum pH for the growth of *Lactobacillus* is 3–4.4, whereas that for *Vibrio cholerae* is 6.0 [25]. Thus pH of honey is unlikely to affect yeast or mold growth. However depending on the type of bacteria and the type of honey, it may be a factor in antibacterial activity. In this study, the pH could not have figured in the antimicrobial activity of honey because the latter was diluted by nutrient broth (pH 7.2). Therefore there must be antimicrobial substance(s) in honey that caused the difference in the antimicrobial activity between honey and the sugar solution.

The antimicrobial substances in honey: Dold et al. [1] described the presence of unknown constituents in honey responsible for its antibacterial activity. They termed these "inhibines". *White* et al. [2] reported that hydrogen peroxide is indeed an "inhibine" and this was emphasized by other authors [3, 4, 7].

Are There Other Antimicrobial Substances in Honey?

Propolis (bee-glue) is a resinous substance collected by bee workers from various plant sources and has wide antimicrobial activity. The flavonoids are the largest group of substances in propolis [26].

Flavonoids exist in many plants. Examples are flavonoids in red grapes [27], in bilberry juice, *Vaccinium myrtillus* [11], in soybeans [28] and in the sour cherry, *Prunus cerasus* [29]. The antimicrobial activity of flavonoids is well documented. Examples are the anti-*Helicobacter pylori* activities of pinocembrin, galanin and chrysin isolated from propolis [30], and the antifungal activities of soybean flavonoids (coumestrol, biochanin A, genistein, naringenin and isorhamnetin) towards *Phytophthora sojae* [28].

Flavonoids have been extracted from honey [31, 32], the most important being pinocembrin, pinobanksin and chrysin [31]. Since honey contains flavonoids and these flavonoids possess antimicrobial activity, they may be considered as one class of inhibines in honey.

The presence of phenolic acids in honey was implied from the similarities between their occurrence and that of flavonoids in nature. Phenolic acids are also present in propolis and in plants. *Azar* et al. [11] separated the phenolic acids (caffeic, chlorogenic, p-coumaric, ferulic, syringic, gallic, protocatechic, p-hydroxybenzoic, m-hydroxybenzoic, vanillic, m-coumaric and o-coumaric acids) from bilberry juice, *Vaccinium myrtillus*. *Cizmarik* and *Matel* [33] separated caffeic acid from propolis. *Cizmarik* and *Matel* [34] isolated ferulic acid from propolis. *Sosulski* et al. [35] separated p-hydroxybenzoic, vanillic, syringic, p-coumaric and ferulic acids from wheat, rice, oats, corn and potatoes.

The phenolic acids also possess antimicrobial activity. *Cizmarik* and *Matel* [33] reported the antimicrobial activity of caffeic acid against *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Proteus vulgaris*, *Mycobacterium tuberculosis*, *Helminthosporum carbonum* and *Streptomyces scabies*. *Cizmarik* and *Matel* [34] showed the antibacterial activity of ferulic acid towards gram-positive and gram-negative bacteria. *Marhuenda Requenda* et al. [36] reported the antibacterial action of caffeic, vanillic, p-coumaric, p-hydroxybenzoic and syringic acids extract of *Thymus carnosus Boiss.*

In this study, caffeic acid and ferulic acid were separated from honey by HPLC and GLC. They had not been separated from honey previously. Other phenolic acids may also be present in honey. These results emphasize that hydrogen peroxide is not the only inhibine present in honey, but it is one of a whole group of antimicrobial substances or inhibines.

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